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ARTICLE

Fruitful female fecundity after feeding *Gryllodes sigillatus* (Orthoptera: Gryllidae) royal jelly

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Abstract

Dietary honey bee royal jelly increases insect growth rates and adult body size. Royal jelly as a dietary supplement could enhance mass insect production by increasing the body size of mass-reared model species. To determine the effect of royal jelly on a cricket species, *Gryllodes sigillatus* Walker (Orthoptera: Gryllidae), farmed for human consumption, we ran two experiments. We tested the dose-dependent response of *G. sigillatus* to royal jelly using a range of diets across 0–30% w/w royal jelly. We also measured the individual-level life history responses to royal jelly over time by individually rearing *G. sigillatus* nymphs on two separate diets: half were fed a commercial cricket diet, and half were fed the same diet mixed with 15% w/w fresh royal jelly. We found sex-dependent effects: females fed the royal jelly diet were 30% heavier, and this effect was driven by significantly longer abdomens containing 67% more eggs compared to those fed the standard diet. Female mass was optimised at approximately 17% w/w royal jelly. Our results reveal that although a royal jelly dietary supplement can increase the yield of mass-reared insects, the life history responses are species and sex specific.

Introduction

Honey bee (*Apis mellifera* Linnaeus) (Hymenoptera: Apidae) royal jelly is a nutrient-rich substance produced and secreted by the hypopharyngeal and mandibular glands of nurse bees that has strong epigenetic influence on the polyphenism of caste development. Honey bee larvae fed royal jelly *ad libitum* will develop into queens, resulting in a caste with remarkable life history differences compared to other caste members, including larger bodies, longer lifespan, and the ability to lay fertilised eggs (Haydak 1943; Ramanathan *et al.* 2018; Slater *et al.* 2020). Over the past decade, the life history–altering effects of royal jelly have been discovered to be interspecific, eliciting changes in body size, feeding, longevity, development time, and fertility in the fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Gardner 1948; Kamakura 2011; Kayashima *et al.* 2012; Shorter *et al.* 2015; Arruda *et al.* 2017), the nematode, *Caenorhabditis elegans* Maupas (Rhabditida: Rhabditidae) (Honda *et al.* 2011, 2015; Detienne *et al.* 2014), the domestic silk moth, *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae) (Hayashiya *et al.* 1965; Nguku *et al.* 2007; Miyashita *et al.* 2016), and the two-spotted field cricket, *Gryllus bimaculatus* DeGeer (Orthoptera: Gryllidae) (Miyashita *et al.* 2016).

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Royal jelly is composed of water (60–70%), proteins (8–18%), carbohydrates (7–18%), fatty acids and lipids (3–8%), and small amounts of vitamins and minerals (Wytrychowski *et al.* 2013; Kunugi and Ali 2019). Royal jelly's variation in nutrient content depends in part on which honey bee species produced it and their geographical location, the timing of harvest, botanical source, storage conditions, pesticide exposure, and production methods (Sano *et al.* 2004; Zheng *et al.* 2011; Wytrychowski *et al.* 2013; Kunugi and Ali 2019; Al-Kahtani and Taha 2020; Chaves *et al.* 2020; Mokaya *et al.* 2020; Qi *et al.* 2020; Ma *et al.* 2021; Milone *et al.* 2021). More than 80% of the protein in royal jelly is in the form of nine major royal jelly proteins (Klaudiny *et al.* 1994; Albert and Klaudiny 2004; Xin *et al.* 2016; Kunugi and Ali 2019). These major royal jelly proteins drive the specific physiological and life history changes that occur during honey bee queen development (Schmitzová *et al.* 1998). Dietary protein is important for providing amino acids required for growth and body-size regulation (Harrison *et al.* 2014; Han and Dingemanse 2017; Reifer *et al.* 2018), and royal jelly contains 17 free amino acids (Silici *et al.* 2009; Ahmad *et al.* 2020), six of which are essential for insects (lysine, valine, threonine, phenylalanine, leucine, and isoleucine; Cohen 2015). Protein is also important for insect egg production (Joern and Behmer 1997; Harrison *et al.* 2014; Roeder and Behmer 2014). Although protein more strongly influences growth than carbohydrates do when diets are provided *ad libitum* (McDonald *et al.* 2011; Harrison *et al.* 2014), the high sugar content – and thus high carbohydrate content – of royal jelly could still affect growth. Sugars are a feeding stimulant that increases the quantity of food consumed (Buttstedt *et al.* 2016; Kunugi and Ali 2019) and thus play an indirect role in body size because individuals who consume more generally tend to grow larger (Gutiérrez *et al.* 2020).

Insects are commonly used as model systems in nutritional ecology due to their short generation times, ease of rearing, and high reproductive output (Gutiérrez *et al.* 2020). These same life history traits make insect species amenable to mass rearing, including being farmed for food and feed. Insects have been consumed around the world for centuries, but insect-farming and mass-rearing facilities are relatively new to Europe and North America. Recently, North America has seen a burgeoning interest in entomophagy (Schrader *et al.* 2016), with 35 new Canadian insect-protein producers and companies offering insect-containing food products in the last decade (Natural Products Canada 2022). Crickets are economically important to these protein-producing companies because these adult insects are more desirable to consumers than larval-stage insects such as mealworms and black soldier fly larvae (Dossey *et al.* 2016; Reverberi 2020).

Advancements in diet technology and formulation could potentially reduce the costs associated with farming insects. Diet can have strong life history influences in insects (*e.g.*, dietary protein and carbohydrates – Harrison *et al.* 2014; Cammack and Tomberlin 2017; Reifer *et al.* 2018; Kim *et al.* 2021; salts – Welti *et al.* 2019; Peterson *et al.* 2021; lipids – Blaul and Ruther 2011; Krabbe *et al.* 2019). Changes in diet formulations and the use of dietary supplements therefore have the potential to enhance yields of insect farms without additional labour. Miyashita *et al.* (2016) discovered that feeding nymphs royal jelly resulted in enlarged body sizes of both male and female crickets, *Gryllus bimaculatus* (Orthoptera: Gryllidae); this effect was dose dependent and optimised at a 15% royal jelly diet. These results suggest that royal jelly, or its component major royal jelly proteins, is a dietary supplement that could enhance cricket farm yields. However, because the effects of dietary royal jelly differ among invertebrate orders and species (Miyashita *et al.* 2016), the dietary requirements for optimal fitness of mass-reared insect species regularly used by the industry may differ from those for *Gryllus bimaculatus*, a species not used in this industry. The time is ripe to translate the effects of royal jelly to increasing the yield of a mass-reared insect model regularly used by industry. We therefore determined the effects of royal jelly on male and female growth rates and adult body sizes of a mass-reared insect to determine whether royal jelly could be used to grow farmed insects bigger and faster.

We ran two experiments using a commercially reared cricket species, *Gryllodes sigillatus* Walker (Orthoptera: Gryllidae), as our model organism. In experiment 1, we asked what minimum amount of royal jelly supplement is required to elicit a significant morphological response. We then proceeded with a more thorough investigation (experiment 2) to determine the individual-level life history responses of *G. sigillatus* to royal jelly over time. We hypothesised that royal jelly would enhance growth in both males and females. Following Miyashita *et al.*'s (2016) findings on *Gryllus bimaculatus*, we predicted that both male and female *G. sigillatus* would grow larger when fed a control diet that included a 15% royal jelly supplement compared with males and females fed a control diet without royal jelly supplement.

Materials and methods

Cricket rearing

Gryllodes sigillatus eggs were hatched in a medium of peat moss inside an incubator (56 × 56 × 99 cm, Precision Scientific Instruments Corp., New Delhi, India) maintained at ~31.5–33 °C and 60% relative humidity. A 14:10-hour light:dark cycle was maintained using a single LED light strip installed along the length of the incubator interior. These conditions were conserved for the duration of both experiments. Every other day, the peat moss and eggs were moistened with water to prevent desiccation and gently stirred to prevent mould growth. The eggs were monitored twice daily for emergence. Upon emergence, only those crickets that emerged within a 12-hour time frame were used for experiments. Newly emerged cricket nymphs were each placed into individual rearing containers made from 96.1-mL plastic condiment cups with aerated lids. Twelve rearing containers were evenly spaced apart in a 4 × 3 grid on plastic cafeteria trays. The trays were assigned a new position inside the incubator every four days, and no tray was assigned the same position for the duration of the experiment. Diet treatments were randomly allocated for each tray. Each cricket was provided with a 14-mm-wide polyethylene push-in cap for a food dish and a 0.75-uL polymerase chain reaction tube (lid removed) for a water vial, which was stoppered with 38-mm-wide saturated dental cotton wick (Healifty, Guangdong Province, China). Food and water were provided *ad libitum* and replaced every 3–4 days. After five weeks, all crickets were euthanised by freezing.

The standard diet (Earth's Harvest Organic Cricket Grower; Earth's Harvest, Oxford Mills, Ontario, Canada) consisted of a mixture of 40.4% corn, 27.9% soybean meal, 15.0% fishmeal, and 1.70% vitamin and mineral mix (NutriMix Elite Grower DDG; Nutri+, Sunnyvale, California, United States of America). Fresh 100% pure royal jelly (Planet Bee Honey Farm, Vernon, British Columbia, Canada) was mixed with the standard diet to make treatment diets consisting of 10, 15, 20, and 30% w/w royal jelly. Diets were stored in a refrigerator at 4 °C.

Measurements

Live cricket individuals were placed into clear plastic Ziploc bags and photographed dorsally using a Dino-Lite Edge 3.0 Digital Microscope (Dunwell Tech, Inc., Torrance, California, United States of America) and DinoCapture 2.0 software (AnMo Electronics Corp., New Taipei City, Taiwan). The camera was calibrated for measurements before photographing specimens, and the subsequent photos were supplemented with a scale bar to accurately perform digital measurements. Head width (maximal distance between the outer edges of the eyes) and pronotum width (maximal distance across the coronal width of the pronotum) and length (maximal distance down the sagittal length of the pronotum) were measured every seven days using ImageJ, version 1.48 software (National Institutes of Health, Bethesda, Maryland, United States of America). Eggs were photographed using the same equipment and methods and were manually counted in ImageJ using the Cell Counter plugin (De Vos, University of Sheffield, Sheffield, United Kingdom).

Experiment 1: dose–response feeding curve

To determine the dose-dependent responses of *G. sigillatus* body size and body mass to royal jelly supplements and the probability of reaching adulthood when fed a royal jelly supplement, we reared *G. sigillatus* nymphs from hatch to adulthood on standard diets with a range of 0–30% royal jelly. Two separate trials were conducted. Upon emergence, cricket nymphs were weighed, photographed, measured, and then placed into individual containers. For trial 1, diets consisting of 0%, 10%, 15%, and 20% royal jelly were fed to 25 crickets per diet ($N = 100$). In trial 2, diets consisting of 0%, 10%, 20%, and 30% royal jelly were fed to 48 crickets per diet ($N = 192$), for a total of 292 crickets in experiment 1 collected across the two trials. Body size and weight measurements were performed 35 days after hatch.

Experiment 2: 15% royal jelly supplement

We tested how adding a 15% royal jelly supplement to the standard diet affected the growth and egg production of *G. sigillatus*. This treatment reflects the dose used by Miyashita *et al.* (2016) and reflects the maximum mass and abdomen responses observed in experiment 1 (Fig. 1). Two separate trials were conducted, and in each trial, 96 crickets were fed either the standard diet with no royal jelly supplement (control; $N = 48$) or the standard diet with a 15% royal jelly supplement ($N = 48$), for a total of 192 crickets in experiment 2. Body mass was measured weekly starting seven days after hatch. Body size and mass measurements were performed weekly for 35 days after hatch, and the crickets were then frozen. We performed *post hoc* measurements on abdomen length (maximal distance down the sagittal length of the abdomen) and the number of eggs produced per cricket ($N = 15$ standard diet; $N = 28$ 15% royal jelly). Abdomens were opened with a ventral longitudinal incision and a smaller perpendicular horizontal incision near the anal opening. The digestive tract was removed, and all eggs were flushed out of the abdominal cavity with water. An image of the eggs was captured, and the number of eggs was counted manually.

Data analysis

All statistical analyses were conducted using JMP Pro, version 15.0.0 (SAS Institute, Cary, North Carolina, United States of America). Normality of the data was confirmed through residual analyses and Shapiro–Wilk goodness-of-fit tests for each variable ($P > 0.05$). We used the false discovery rate B–Y method (FDR_{BY}) to adjust *alpha* for each statistical test (Benjamini and Yekutieli 2001). "Trial" was included as a random effect in all analyses and was never statistically significant ($P > 0.05$).

Experiment 1: dose–response feeding curve

Data from experiments 1 (0%, 10%, 15%, 20%, and 30% royal jelly supplements) and 2 (0% and 15% royal jelly supplements) were pooled to increase sample size, for a total of 484 crickets ($N = 169$, 73, 121, 73, and 48 for the 0, 10, 15, 20, and 30% royal jelly diets, respectively). In total, 304 crickets survived, and 280 reached adulthood (see Supplementary material, Table S1 for survival distribution). Crickets that did not reach adulthood by the end of the experiment were removed from the analyses. We used logistic regression to explore how royal jelly supplements affected the probability that individual crickets would reach adulthood. The categorical dependent variable was whether the individual was a juvenile or an adult at

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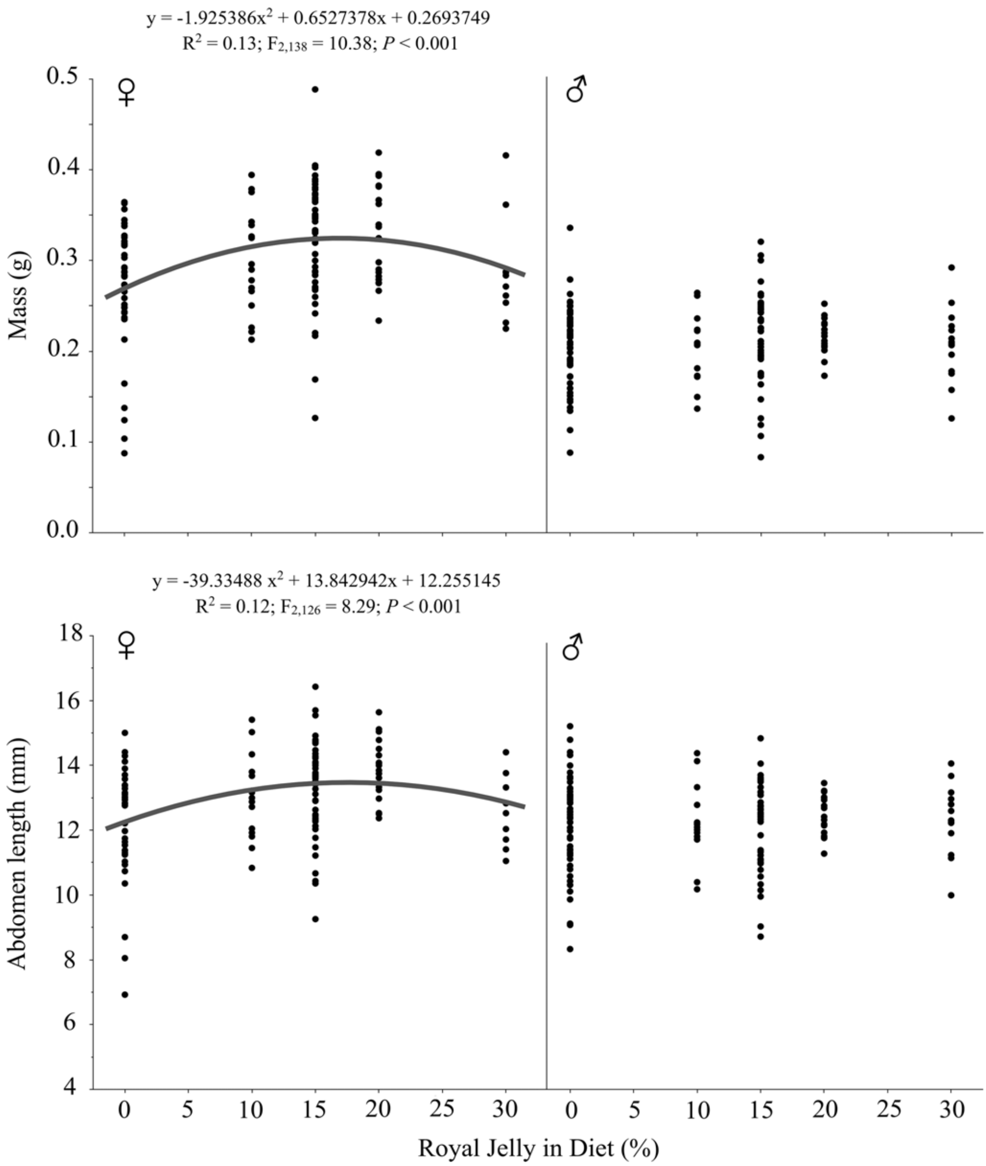

Fig. 1. Royal jelly dose–response regressions of *Gryllodes sigillatus* ($N = 169$, 73, 121, 73, and 48 for the 0%, 10%, 15%, 20%, and 30% royal jelly diets, respectively). Female mass and abdomen length are explained by significant quadratic relationships.

35 days after imaginal moult, and the independent variable was the percentage of royal jelly (w/w) in the diet.

To determine whether royal jelly concentration impacted adult mass and body size, we used linear mixed models. If significant linear and quadratic regressions were found for a variable, sample-size adjusted Akaike information criterion values (AICc) were used to select the best fit. Diet (0%, 10%, 15%, 20%, and 30% royal jelly) and sex (female or male) were included as fixed effects, and experiment trial (experiment 1: trials 1 and 2; experiment 2: trials 3 and 4) was included as a random effect.

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Experiment 2: 15% royal jelly supplement

A total of 161 of 192 crickets reached adulthood in experiment 2. Crickets that did not reach adulthood by the end of the experiment were removed from the analysis. Of the surviving 161 crickets, 45 males and 33 females were fed the standard diet and 36 males and 47 females were fed the royal jelly diet. A chi-squared test was performed to determine if these ratios significantly differed from those expected. Principal component analysis was used to extract orthogonal vectors from head width, pronotum width, and pronotum length at adulthood to quantify adult body size into a single measurement. The first principal component explained 92.12% of the variation in size (eigenvalue = 2.76); all size measures loaded equally on the first principal component. We used a linear mixed model to determine whether diet treatment influenced mass, body size (first principal component), head width, pronotum width, pronotum length, and abdomen length. In all analyses, diet (standard diet only – the control – or standard diet with 15% royal jelly supplement), age (days since hatch), sex (male or female), and all possible interactions were included as fixed effects. Given the repeated aspects of the experiment, both cricket identification number and trial were included as random effects. We used a linear model to determine whether royal jelly affected day-of-eclosion adult mass, body size (first principal component), head width, pronotum width, pronotum length, and abdomen length. Diet, sex, and the interaction between diet and sex were included as fixed effects, and trial was included as a random effect. A separate linear model was used to determine whether royal jelly affected the number of eggs at adulthood, with diet as the only effect in the model because only females produced eggs and eggs were dissected only in trial 1. Pairwise comparisons for the linear model were developed using Tukey's honestly significant difference test.

Results

Dose–response feeding curve

The odds ratio of crickets reaching adulthood after 35 days was 19.2 higher for crickets fed royal jelly ($X^2 = 14.27$, $df = 1$, $P < 0.001$, $R^2 = 0.12$). Significant linear and quadratic relationships were found between royal jelly concentration and female mass and abdomen length (Table 1; Fig. 1), and the quadratic relationships both fit better than the linear relationships, based on the lower AICc values. The quadratic relationships between royal jelly and female mass and abdomen length predicted maximised responses when fed 16.95% and 17.60% royal jelly, respectively. Summary statistics from the linear mixed model are provided in Supplementary Material, Table S2.

15% Royal jelly supplement

The ratio of crickets that survived and developed into males and females was significantly different than expected ($X^2 = 3.89$, $df = 1$, $P = 0.049$). However, this result was made less clear after pooling the 0% and 15% data across the two experiments and running the same chi-squared test, with no difference found between expected and observed ratios ($X^2 = 2.34$, $df = 1$, $P = 0.13$). The 15% royal jelly supplement significantly affected *G. sigillatus* mass ($P < 0.001$) and pronotum length ($P = 0.025$), but males and females were affected differently (Table 2). Females grew heavier ($P = 0.031$) when fed a royal jelly supplement, but the royal jelly supplement did not significantly affect overall body size as encapsulated by the first principal component ($P = 0.07$; Table 2). Over time, females increased mass, grew larger bodies (first principal component), and grew longer pronotums more rapidly when they were fed 15% royal jelly supplements compared to the standard diet without supplements ($P < 0.05$; Table 2; Fig. 2). At day 35, adult females fed royal jelly were 30% heavier on average, had

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Table 1. Summary statistics from the linear mixed model of 35-day-old adult *Gryllodes sigillatus* measurements from experiment 1: dose–response feeding curve.

Model	R^2	Term	*df*	*F*	*P*
Mass	0.53	**Diet**	**1, 272.60**	**15.68**	**<0.001**
		Diet²	**1, 274.00**	**5.17**	**0.030**
		Sex	**1, 271.20**	**215.57**	**<0.001**
		Diet × sex	**1, 271.60**	**7.43**	**0.011**
		Diet² × sex	**1, 271.50**	**4.37**	**0.038**
Body size (PC1)	0.49	Diet	1, 270.70	0.90	0.570
		Diet²	1, 271.90	0.038	0.850
		Sex	**1, 270.10**	**167.45**	**<0.001**
		Diet × sex	1, 270.20	0.92	0.570
		Diet² × sex	1, 270.10	0.31	0.720
Abdomen length	0.26	**Diet**	**1, 257.60**	**8.94**	**0.008**
		Diet²	**1, 258.80**	**4.42**	**0.037**
		Sex	**1, 256.20**	**21.36**	**<0.001**
		Diet × sex	**1, 256.50**	**7.57**	**0.011**
		Diet² × sex	**1, 256.40**	**5.08**	**0.031**

Terms in bold are statistically significant.

significantly longer abdomens, and had significantly higher fecundity (66.6% more eggs) than did females fed the standard diet (Tables 3 and 4; Fig. 3). Male mass and body size were unaffected by royal jelly both over time and at the end of the experiment (Figs. 2 and 3). Trial was never a significant source of variation ($P > 0.05$). Summary statistics from the linear model are provided in Supplementary Material, Table S3.

Discussion

Evidence from several insect orders and nematodes suggests that honey bee royal jelly, when administered orally through diet, can influence life history trait development. Key similarities among insects include increased body size and mass (*B. mori* – Hayashiya *et al.* 1965; Nguku *et al.* 2007; *D. melanogaster* – Kamakura 2011; Shorter *et al.* 2015; Miyashita *et al.* 2016; Arruda *et al.* 2017; *Gryllus bimaculatus* – Miyashita *et al.* 2016) and increased fecundity (*D. melanogaster* – Kamakura 2011; Kayashima *et al.* 2012; *B. mori* – Miyashita *et al.* 2016). Despite this body of past work, the present study is the first to our knowledge that tests the effects in an insect species mass reared for food and feed. We found a sex-specific effect of royal jelly in a hemimetabolous insect (*G. sigillatus*): females grew heavier more quickly and developed more eggs when fed royal jelly, but males were unaffected. These results complicate our understanding of the effects of royal jelly in insects because they both complement and contrast with Miyashita *et al.*'s (2016) finding that both male and female *Gryllus bimaculatus* – a cricket in the same family as *G. sigillatus* – grew larger when fed royal jelly. In the same study by Miyashita *et al.* (2016), sex-specific differences similar to those we observed in the present study were found in the lepidopteran *B. mori*; female moth pupae and adults grew larger and developed more eggs when fed royal jelly. Miyashita *et al.* (2016) suggested that the

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Table 2. Summary statistics from the repeated measures linear mixed model of *Gryllodes sigillatus* measurements from experiment 2: direct test of 15% royal jelly.

Model	R^2	Term	*df*	*F*	*P*
Mass	0.84	**Diet**	**1, 153.3**	**11.74**	<**0.001**
		Sex	**1, 153.3**	**37.23**	<**0.001**
		Age	**1, 712.1**	**3533.24**	<**0.001**
		Diet × sex	**1, 153.4**	**4.75**	**0.031**
		Diet × age	**1, 732.9**	**16.71**	<**0.001**
		Sex × age	**1, 732.9**	**102.34**	<**0.001**
		Diet × sex × age	**1, 732.9**	**12.04**	<**0.001**
Body size (PC1)	0.96	Diet	1, 156.0	4.08	0.063
		Sex	**1, 156.0**	**9.33**	**0.0062**
		Age	**1, 797.5**	**17054.58**	<**0.001**
		Diet × sex	1, 156.1	3.58	0.070
		Diet × age	1, 797.5	1.76	0.190
		Sex × age	**1, 797.5**	**86.65**	<**0.001**
		Diet × sex × age	**1, 797.5**	**4.86**	**0.049**
Head width	0.96	Diet	1, 156.0	2.68	0.100
		Sex	**1, 156.0**	**4.75**	**0.031**
		Age	**1, 797.4**	**19372.19**	<**0.001**
		Diet × sex	1, 156.1	2.94	0.088
		Diet × age	1, 797.4	0.63	0.430
		Sex × age	**1, 797.4**	**73.49**	<**0.001**
		Diet × sex × age	1, 797.4	2.80	0.094
Pronotum width	0.96	Diet	1, 156.1	4.14	0.060
		Sex	**1, 156.0**	**8.71**	**0.0085**
		Age	**1, 797.5**	**17363.46**	<**0.001**
		Diet × sex	1, 156.2	3.52	0.073
		Diet × age	1, 797.5	2.79	0.096
		Sex × age	**1, 797.5**	**80.90**	<**0.001**
		Diet × sex × age	1, 797.5	4.25	0.061
Pronotum length	0.94	**Diet**	**1, 155.9**	**5.70**	**0.025**
		Sex	**1, 155.9**	**17.43**	<**0.001**
		Age	**1, 797.5**	**11711.02**	<**0.001**
		Diet × sex	1, 156.0	4.18	0.050
		Diet × age	1, 797.5	1.99	0.160
		Sex × age	**1, 797.5**	**92.92**	<**0.001**
		Diet × sex ×age	**1, 797.5**	**7.58**	**0.011**

Terms in bold are statistically significant.

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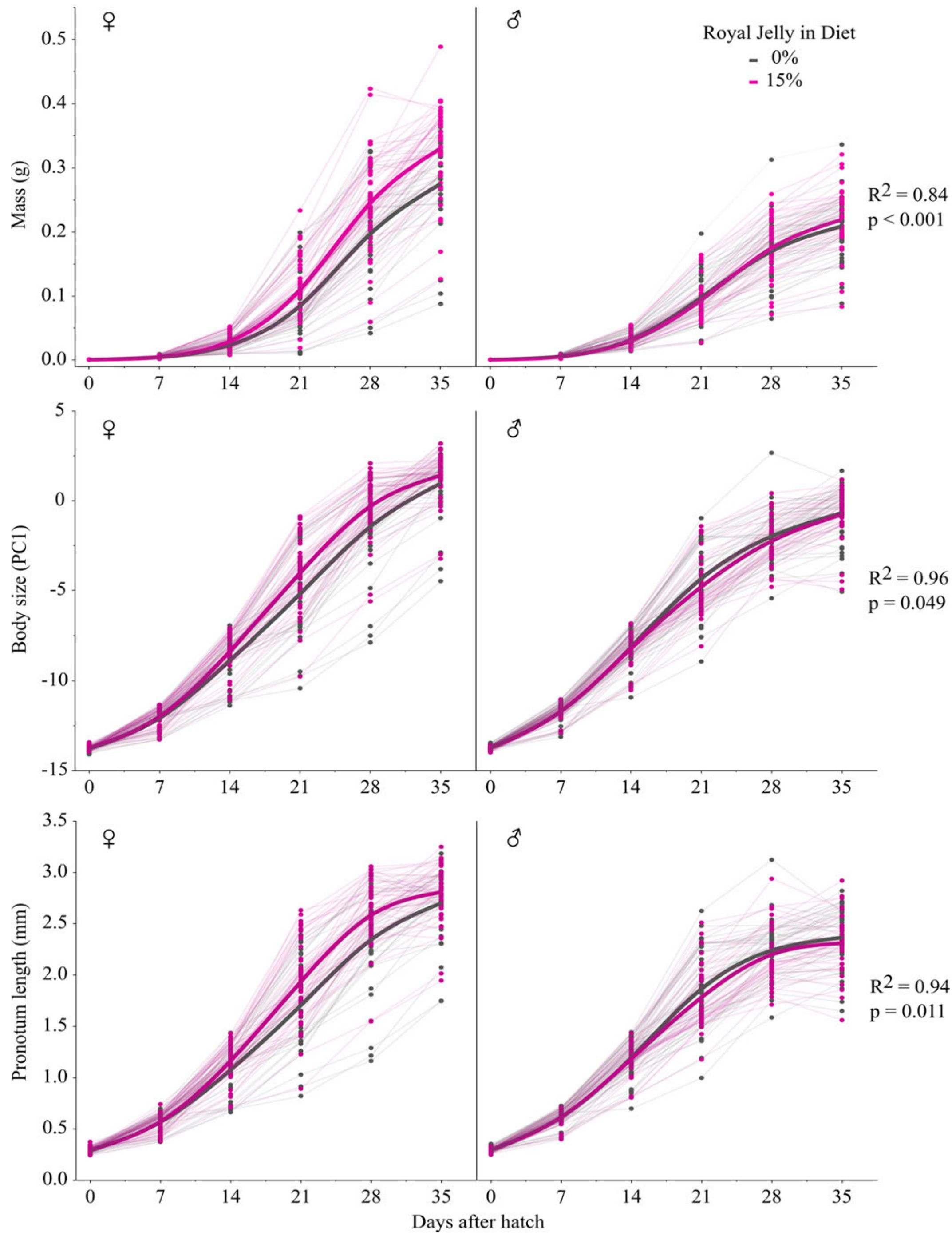

Fig. 2. Lifetime development of mass, body size (PC1), and pronotum length over age of *Gryllodes sigillatus* fed either a standard diet ($N = 80$) or a diet with 15% royal jelly ($N = 84$). Smoothed thick lines connect mean values at each time point, and solid circles represent individual crickets and are connected by thin lines over time.

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Table 3. Least square means ± standard error of morphology and life history measurements of 35-day-old adult *Gryllodes sigillatus* from experiment 2: direct test of 15% royal jelly. Values within a column followed by different letters are significantly different ($P < 0.05$).

Sex	RJ%	Mass (g)	Body size (PC1)	Head width (mm)	Pronotum width (mm)	Pronotum length (mm)	Abdomen length (mm)	Number of eggs
Female	0	0.27 ± 0.023 b	0.95 ± 0.70 a	3.45 ± 0.11 a	4.21 ± 0.12 a	2.70 ± 0.19 a	12.25 ± 0.71 b	151.53 ± 23.92 b
Female	15	0.33 ± 0.022 a	1.27 ± 0.69 a	3.48 ± 0.11 a	4.31 ± 0.12 a	2.77 ± 0.19 a	13.27 ± 0.70 a	250.86 ± 18.32 a
Male	0	0.21 ± 0.022 c	−0.80 ± 0.69 b	3.18 ± 0.11 b	3.79 ± 0.12 b	2.33 ± 0.19 b	12.02 ± 0.70 b	
Male	15	0.22 ± 0.023 c	−0.72 ± 0.70 b	3.19 ± 0.11 b	3.84 ± 0.12 b	2.33 ± 0.19 b	12.20 ± 0.72 b	

Notes: RJ, royal jelly; PC1, first principal component.

Table 4. Summary statistics from the linear model of 35-day-old adult *Gryllodes sigillatus* measurements from experiment 2: direct test of 15% royal jelly.

Model	R^2	Term	*df*	*F*	*P*
Mass	0.51	**Diet**	**1, 156**	**15.45**	<**0.001**
		Sex	**1, 156**	**103.95**	<**0.001**
		Diet × sex	**1, 156.2**	**4.25**	**0.041**
Body size (PC1)	0.45	Diet	1, 156	0.97	0.490
		Sex	**1, 156**	**79.97**	<**0.001**
		Diet × sex	1, 156.1	0.31	0.580
Abdomen length	0.25	**Diet**	**1, 142.1**	**6.20**	**0.021**
		Sex	1, 142	7.24	**0.021**
		Diet × sex	1, 142.1	3.01	0.085
Number of eggs	0.18	Diet	1, 44	10.87	**0.002**

PC1, first principal component.
Terms in bold are statistically significant.

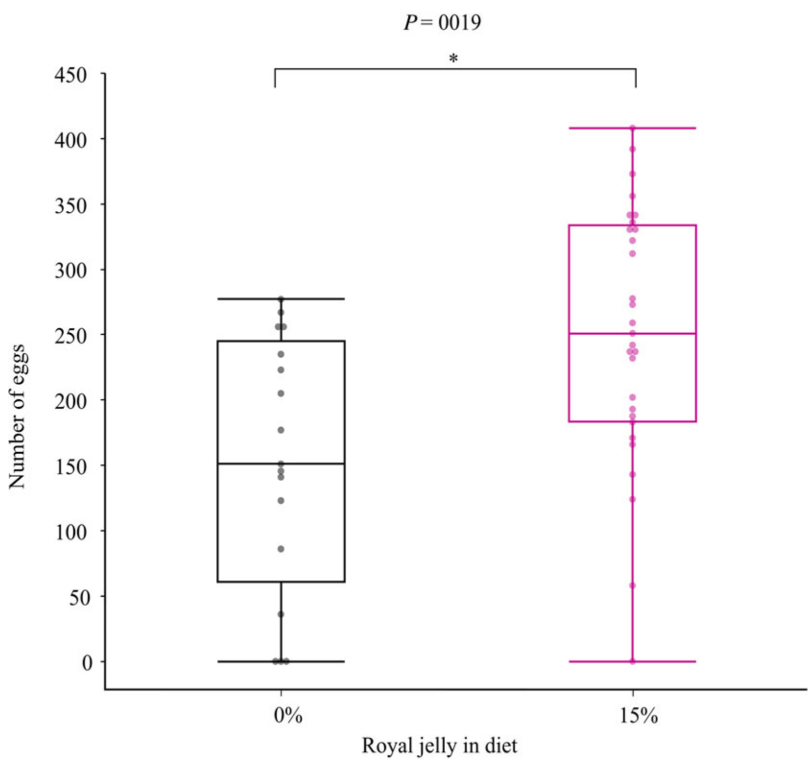

Fig. 3. Number of eggs dissected from 35-day-old adult *Gryllodes sigillatus* females fed standard ($N = 17$) and 15% ($N = 29$) royal jelly diets. Auto jitter was applied to the data points in JMP Pro Graph Builder. The middle line of each box represents the median and the outer edges represent the maximum and minimum values.

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response differences to royal jelly observed in *B. mori* and *Gryllus bimaculatus* were a result of different developmental processes and mechanisms that respond to royal jelly between holometabolous and hemimetabolous insects. Although the theory of insects with different development life histories responding differently to diet is not new (Bernays 1986; Thompson 2019; Neiro 2020; Bertram *et al.* 2021), our results suggest that the effects of royal jelly can be sex specific in Orthoptera. Thus, distantly related holometabolous and hemimetabolous insects can respond similarly to dietary royal jelly, whereas two relatively closely related hemimetabolous orthopterans can respond differently. The signalling mechanisms activated by royal jelly molecules may thus be conserved between holometabolous and hemimetabolous insects, but the ultimate effects of royal jelly may be tied to other life history traits or the dietary or environmental context in which it is experienced by an insect. Miyashita *et al.* (2016) concluded that royal jelly affected cricket size *via* upregulation of food consumption; although this would have been an excellent addition to our study, limitations brought on by the COVID-19 pandemic severely restricted access to resources necessary to accurately measure food consumption.

Sex-specific life history differences in response to diet are well documented in insects, especially in crickets. Protein intake is important for optimising fecundity and egg production in females, and carbohydrate intake regulates calling effort in males (*Teleogryllus commodus* Walker – Maklakov *et al.* 2008; *Gryllus veletis* Alexander and Bigelow – Harrison *et al.* 2014; *T. oceanicus* Le Guillou – Ng *et al.* 2018; *Gryllus assimilis* (Fabricius) – Reifer *et al.* 2018). Houslay *et al.* (2015) provide a stimulating discussion on the differences in resource acquisition between females and males; females have a straightforward process of gathering resources and converting them into eggs, whereas males exhibit acquisition-related plasticity and must choose how to allocate invested resources (calling effort, competition, *etc.*). The investment strategy of male *G. sigillatus* may explain the differences between sexes in the present study; males exhibit acquisition-related plasticity in calling behaviour (Bertram *et al.* 2011; Houslay *et al.* 2015), and therefore, compared to females, which become fecund by gathering nutrients and converting those nutrients into eggs, males experience the resource-intensive additional burden of producing a spermatophylax and conserve resources and energy until they perceive a competitor (Mallard and Barnard 2003, 2004). Investment strategy and resource acquisition may also explain the response differences to royal jelly by *G. sigillatus* and *Gryllus bimaculatus*. Both species increase the number of sperm transferred in the presence of apparent competitors; male *G. sigillatus* significantly increases sperm with a conspecific competitor, whereas *Gryllus bimaculatus* increases sperm regardless of the competitor species (Mallard and Barnard 2003). In a group environment rearing protocol as established by Miyashita *et al.* (2016), it is no surprise that both male and female *Gryllus bimaculatus* increased body size on a royal jelly (*i.e.,* higher-quality) diet; males were susceptible to competition and likely increased their investment by consuming more food, and females grow heavier under higher population density (El-Damanhouri 2011). Under the isolated and individual-rearing conditions used in the present study, we suspect that *G. sigillatus* were less able to detect competition, and therefore no competitor cue increased the crickets' investment in body size. This hypothesis could be tested by repeating our experiment in a controlled group-rearing and active farm environment to determine whether royal jelly also influences body size of male *G. sigillatus* in a group environment. If *G. sigillatus* were fed royal jelly in a mass-rearing environment with a constant presence of conspecific competition, males may demonstrate a response of increased body size regulated by increased investment.

In addition to farm-scale trials, future research should also explore how royal jelly – either fresh or manufactured – impacts male and female growth rates and body size over generations. Warbrick-Smith *et al.* (2009) showed that lab populations of *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) reared on high-carbohydrate diets initially responded with reduced fitness; however, over 350 generations, they evolved the ability to reduce fat storage.

Hall *et al.* (2008) reared Australian ground crickets, *Pteronemobius* sp. Jacobson (Orthoptera: Trigonidiidae) for seven generations on low- and high-quality diets, and although individuals at the beginning of the experiment grew heavier on the high-quality diet, after seven generations, crickets grew significantly heavier on the low-quality diet treatment. Based on these examples, single-generation diet changes may be misleading in their long-term effects but are still valuable for demonstrating the potential power that dietary supplements may have. Further research should test royal jelly across multiple generations of *G. sigillatus* to determine how it affects overall farm yield and whether such supplements would be financially viable for use in insect farming.

Our study is useful in its contrast to Miyashita *et al.*'s (2016) work regarding species-specific nutritional requirements to optimise fitness. Our study also represents an important step towards application of nutritional supplements in cricket farming. *Gryllodes sigillatus* is an important economical cricket species currently being farmed for food and feed in North America. Small- and medium-scale cricket farms often use whatever feed is safe, available, and consumed by the insects: if the crickets eat, grow, and reproduce, the feed is deemed acceptable (Lee Bess, Entomo Farms, personal communication). This keeps production costs low but can lead to lower harvest yields and variable nutrient quality of the food products (Weru *et al.* 2021). Our results contribute to a growing body of literature that demonstrates the strong potential of using royal jelly as a dietary supplement to optimise the mass rearing of insect species. Although royal jelly is expensive, difficult to produce in large quantities (Ramanathan *et al.* 2018), and quick to spoil in storage (Tarantilis *et al.* 2012), research into improving the mass production of natural and artificial royal jelly proteins responsible for enhancing body size and growth is underway (Cao *et al.* 2016; Wang *et al.* 2020; Ma *et al.* 2021). Synthesising royal jelly into mass-reared insect diets must be cost effective but also maintain or increase the gustatory appeal of the diet. Royal jelly is rich in free amino acids (Guo *et al.* 2021), and female *G. sigillatus* feeding time increases with spermatophylax amino acid concentration (Warwick *et al.* 2009), thereby increasing the total amount of ejaculate transferred to females. Diet can have a strong influence on male *G. sigillatus* fitness (Duffield *et al.* 2020), and male mating success is maximised on diets that are slightly higher in protein than carbohydrates (Rapkin *et al.* 2017). Future studies testing how dietary royal jelly influences spermatophylax composition, feeding time, and overall mating success in *G. sigillatus* would therefore be valuable.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.4039/tce.2022.39.

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Declaration of competing interest. M.J.M, H.A.M., and S.M.B. have a research partnership with Entomo Farms.

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